Ethoxysulfuron Causes Morphological Damages, DNA damage, Oxidative Stress and Antioxidant Enzyme Activity in Fresh Water Fish

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Abstract- Ethoxysulfuron, herbicide is used to control undesired plants but can lead to contamination of aquatic ecosystems. This research was conducted to observe the toxic impacts of Ethoxysulfuron on Freshwater Rohu (*Labeo rohita*). The main object of study was to assess the genotoxic potential and biomarkers of oxidative stress and the status of antioxidant enzymes in *Labeo rohita* exposed to different concentration of Ethoxysulfuron. For this purpose, 60 fresh water fishes (*Labeo rohita*) were purchased with no clinical ailments from fish hatchery in Bahawalpur Pakistan. The fish were kept in glass aquarium and allowed to acclimatize for a week. The temperature was kept almost 28 to 32°C. Constant air was bubbled into aquarium by air pump. The fish were divided into four groups (A-D), with Group A serving as the control, and Groups B, C, and D receiving 0.250g/L, 0.375g/L, and 0.500g/L of Ethoxysulfuron, respectively, over 21 days. Water temperature, pH, dissolved oxygen and mortality of fish were noticed during experiment after every 12 h. we collected various tissues, including blood and visceral organs (heart, brain, liver, gills, kidneys, and blood), from each fish after 7 ,14 and 21days respectively. The results show that significant genotoxic effects in treated groups, including increased micronuclei and various nuclear abnormalities in erythrocytes. Morphological changes in RBCs were noticed, including decreased RBC values and increased leptocytes, spherocytes, microcytes, and pear-shaped erythrocytes. Additionally, biomarkers of oxidative stress were increased, while antioxidant enzyme levels were decreased in treated fish. These findings suggest that Ethoxysulfuron produce oxidative stress and genotoxicity, adversely affecting multiple tissues in *Labeo rohita*, and point out the potential environmental risks of this herbicide to aquatic organisms.

Key Words- *Labeo rohita*, Ethoxysulfuron, DNA damage, Aquatic Toxicity, Oxidative Stress

1. INTRODUCTION

Fish and other aquaculture species are crucial in the global fight against hunger both humans and other terrestrial animals. If food security and supply cannot be maintained due to the rapid development of the human population, it may result in a variety of issues with relation to the consumption of nutritious foods. Therefore, in order to meet the demands of an expanding human population in a number of developing countries, optimal aquaculture production and health are now required [1]. Large amounts of protein are generated through global aquaculture. The aquaculture business is under pressure from the ever-growing human population, water shortages, the high cost of land acquisition, and the careless use of many different chemicals and medications [2]. The use of pesticides has a variety of major and minor benefits, with the most well-articulated being financial gains from the assurance of agricultural output, quality, and the decrease of many extra expensive sources, such fuel and labor [3]. Over 80% of all pesticides used in Pakistan are used on the cotton crop, which is a crucial cash crop and a vital component of the total national economy [4]. Almost 90% of all pesticides found in nature are insecticides. Approximately 5 various types of acaricides, 6 different types of rodenticides, 30 various kinds of fungicides, 39 various kinds of weedicides, and far more than 108 various kinds of insecticides are used in Pakistan [5]. Residues of these compounds in water, food, crops, and ecosystems can seriously intimidate organisms, including predators and the human population [6]. All pesticides are recognized as major water pollutants that seriously disrupt the aquatic ecology [7]. There are three main ways that fish and aquatic animals are exposed to pesticides. direct skin to-skin contact with pesticides while swimming in contaminated waters, Pesticides disbursed vocally through digestion of pesticides [8]. Numerous studies have shown that people who use pesticides frequently experience a variety of health issues, such as skin issues [9]. Stress, respiratory issues, hematological and biochemical damages, coronary artery disease, and cancer. According to several studies on the Stress of pesticide merchants, exposure to active pesticide ingredients may stimulate various adverse health outcomes in this working community [10]. The highly efficient sulfonylurea herbicide Ethoxysulfuron, technically known as 3-(4, 6- dimethoxyoyrimidin-2-yl)-1-(2-ethoxyphenoxysulfonylurea), is used as a selective herbicide against particular weeds. There is less information known about the toxicity of Ethoxysulfuron. This type of pesticide, its solubility, its concentration in the medium, the quantity of algal cells, and the size of the cells all affect how much pesticide builds up. By changing the species mix of an algal community, herbicides can have an impact on the structure and operation of aquatic communities [11]. Sea creatures exposed to various pollutants may experience physical stress that leads to mutagenic abnormalities and structural nuclear alterations in their erythrocytes [12]. Studies show that examining the morphological characteristics and nuclear alterations of erythrocytes is commonly used in noxiousness testing and to assess the health of fish [13]. Currently, there is a limited amount of information

regarding the potential toxicity of Ethoxysulfuron. The characteristics of the pesticide, such as its solubility and concentration within the environment, as well as the abundance and size of algal cells present, all contribute to the accumulation of the pesticide [14]. Aquatic species regularly employ the comet assay to measure DNA damage, which is of enormous value [15].

Objectives: Due to limited studies on Ethoxysulfuran pesticide, main objective to evaluate the Ethoxysulfuron exposure of oxidative stress levels, antioxidant enzyme activity, Genotoxic potential of Ethoxysulfuron in fish, study morphological changes in RBCs and to study Nuclear changes in fish.

2. Methodology

Fish management and Maintenance of experimental laboratory

A total number of 60 rohu fish (*Labeo rohita*) purchased from fisheries complex. Fish were transported to the laboratory within plastic bag containing oxygen and water. All of these fish were of uniform size and age. Weight of fish were measured; up to 25-120 g. when transferred to the zoology laboratory of the Islamia university of Bahawalpur. In the glass aquarium all trial fishes were moved having 100-liter water and kept under normal Lab circumstances, acclimatized for 4 days. Before the arrival of fishes' whole aquarium were properly washed before water filling and arrival of trial fishes. The water temperature was between 28°C-32°C. The pH of water was 7 ± 1. There were windows in Lab for suitable aeration. Lights facility in the Lab was also maintained.

Procedure

Fish in the Control group in this experiment received daily feedings of a normal diet. While the other groups received different doses of Ethoxysulfuron

Table: Shows different concentration of Ethoxysulfuron applied on experimental fish

Groups	*Concentration g/l*
A	0
B	0.25
C	0.375
D	0.50

Blood Sampling and Organ Collection and Storage

1st Blood samples were collected from 12 fishes per group using a 3ml disposable syringes with a needle from caudal of fish and dipped in EDTA to prevent coagulation and preserve blood cells morphology. Approximately 1.2ml of blood was taken from each fish's caudal vein and stored in separate EDTA blood tubes for future hematological analysis. Then visceral organs such as gills, brain, heart, liver, and kidney were carefully dissected from fish and stored at 4°C in aluminum foil for further analysis of oxidative stress and antioxidant enzyme activities.

Oxidative Stress and Antioxidant Enzyme Studies

Fish organs (gills, liver, kidney, heart, brain) were dissected, homogenized in 0.2M cold phosphate buffer (1:4 w/v), and centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatants were stored at -80°C for biochemical analysis.

TBARS Assay: A reaction mixture was prepared and boiled at 100°C for 15 minutes. Absorbance was measured at 532nm.

CAT Assay: A mixture of H_2O_2 solution, PBS (pH 5), and the sample was prepared. Absorbance was measured at 240nm.

GSH Assay: The reaction mixture included the sample, potassium phosphate buffer, and DTNB reagent. Absorbance was noted at 412nm.

ROS Assay: A reaction mixture of sodium acetate buffer, $FeSO_4$ + DEPPD solution, and the tissue sample was prepared. Absorbance was measured at 505nm.

POD Test: The reaction mixture included H_2O_2, potassium phosphate buffer, guaiacol solution, and the tissue sample. Absorbance was measured at 470nm.

SOD Test: A mixture of methionine, NBT, Triton, and phosphate buffer was prepared. After exposure to light and incubation with riboflavin, absorbance was measured at 560nm.

Hematological Evaluation

A small drop of blood from each sample was placed on one end of a glass slide, and another clean slide was used to spread the blood into a smear. After air drying, the blood films were fixed with methanol and stained with Giemsa stain. The stained slides were then washed with distilled water and examined under a fluorescence microscope at 100x magnification.

Genotoxicity Assessment using the micronuclei assay and comet assay

Micronuclei Assay: Blood cells were stained with Giemsa to observe morphological changes at the nuclear level.

Comet Assay: This assay was conducted following the method by Singh et al. (1988) with modifications. Key solutions prepared included:

- Phosphate Buffer Solution (PBS): pH adjusted to 7.4.
- Lysing Solution: pH adjusted to 10.
- Electrophoresis Buffer Solution: pH adjusted to 13.
- Neutralization of Solution: pH adjusted to 7.5.
- Staining Solution: 10mg of Ethidium Bromide in 50ml of deionized water.

Slide Preparation: Three layers were prepared on frosted slides:

i. First Layer: 90µl of 1% normal melting agarose in PBS, solidified at 4°C.

ii. Second Layer: Leukocytes mixed with 0.5% low melting agarose, solidified at 4°C.

iii. Third Layer: 90µl of 1% normal melting agarose, solidified at 4°C.

Treatment of Slides:

• Lysing: Slides dipped in chilled lysing solution at 4°C for 1 hour.

• Electrophoresis: Conducted at pH 13 for 25 minutes at 25V, followed by neutralization.

• Staining: Slides stained with Ethidium Bromide and analyzed using an epi-fluorescence microscope at 400x magnification.

Statistical Analysis

We are using one-way ANOVA for getting statical values under totally randomized design, values for different oxidative stress and antioxidant enzymes Mean+SE calculated and dissimilar group means were contrast by Tukey's test with ($P \leq 0.05$). Also, Duncan post-hoc test used after repeated measures analysis of variance to determine the statistical significance of group differences.

3. Results

Morphological changes in erythrocytes Pear shaped erythrocyte (%): The percentages of fish with pear-shaped erythrocytes in the treatment groups are shown in **fig. 4.1.** After 14 and 21 days of Ethoxysulfuron poisoning, there was a considerably ($P \leq 0.05$) higher percentage of pear-shaped erythrocytes (%) in fish from Groups C and D.

Leptocytes (%): The percentages of fish leptocytes in the treatment groups shown in **Fig. 4.2**. After being exposed to

Ethoxysulfuron for After 7, 14, 21 days, the levels of leptocytes (%) in fish from Groups B, C, and D significantly (0.05) increased.

Spherocytes (%): The percentages of fish spherocytes in the treated groups shown in **Fig. 4.3**. After 7, 14 and 21 days of Ethoxysulfuron poisoning, there was a 31 substantial (P≤0.05) increase in the percentage of spherocytes in fish from Groups B, C, and D.

Microcytes (%): The percentages of fish with microcytes that were present in the treated groups shown in **Fig. 4.4**. After 7, 14 and 21 days of Ethoxysulfuron poisoning, the levels of microcytes (%) in fish from Groups B, C, and D significantly (0.05) increased.

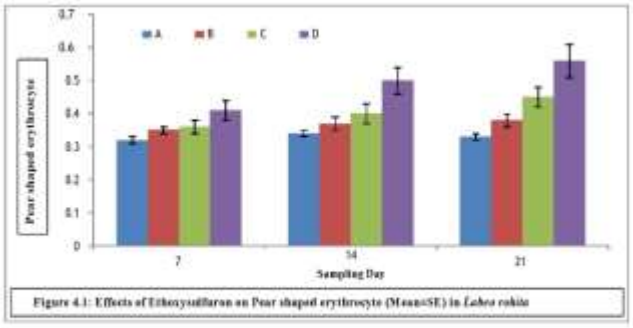


Figure 4.1: Effects of Ethoxysulfuron on Pear shaped erythrocyte (Mean±SE) in *Labeo rohita*

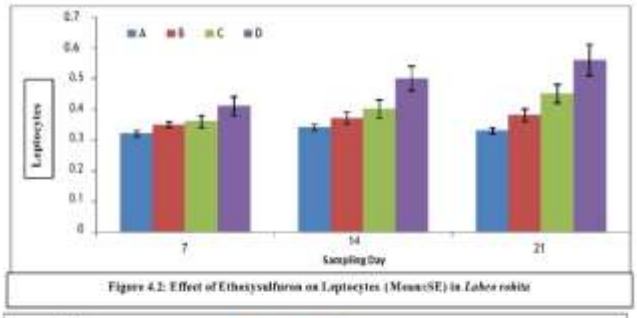


Figure 4.2: Effect of Ethoxysulfuron on Leptocytes (Mean±SE) in *Labeo rohita*

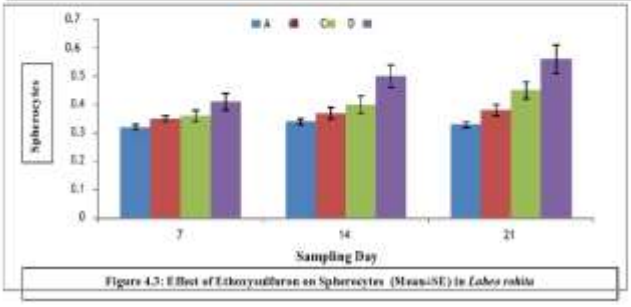


Figure 4.3: Effect of Ethoxysulfuron on Spherocytes (Mean±SE) in *Labeo rohita*

Nuclear changes in erythrocytes

Erythrocyte with broken nucleus (%): The percentages of fish treatment group erythrocytes with damaged nuclei **shown fig. 4.5.** After 7, 14 and 21 days of Ethoxysulfuron poisoning, there was a substantial (P≤0.05) increase ratio of erythrocytes with fragmented nuclei in fish belonging to Group C and D.

Erythrocyte with lobed nucleus (%): The percentages of fish erythrocytes with lobed nuclei that were present in the treatment groups shown **figure (4.6).** After 7, 14 and 21 days of Ethoxysulfuron poisoning, fish in Groups C and D had considerably (P≤0.05) higher percentages of erythrocytes with lobed nuclei (%). erythrocytes with a lobed nucleus value.

Erythrocyte with micronucleus (%): The percentages of fish erythrocytes with micronuclei that were present in the treatment groups shown in **figure (4.7).** The findings showed that after 7, 14 and 21 days of Ethoxysulfuron poisoning, the values of erythrocytes with micronucleus (%) in fish from Group C and D were substantially (0.05) higher.

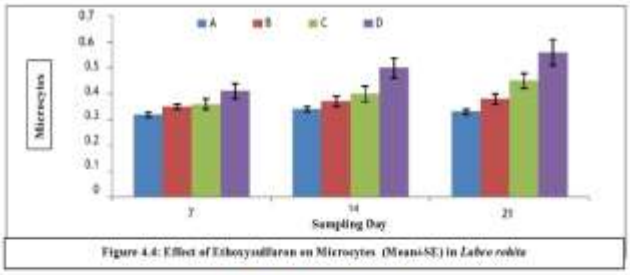


Figure 4.4: Effect of Ethoxysulfuron on Microcytes (Mean±SE) in *Labeo rohita*

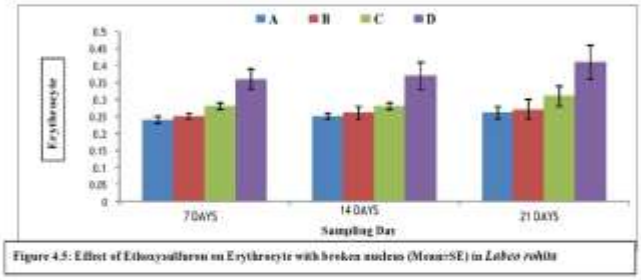


Figure 4.5: Effect of Ethoxysulfuron on Erythrocyte with broken nucleus (Mean±SE) in *Labeo rohita*

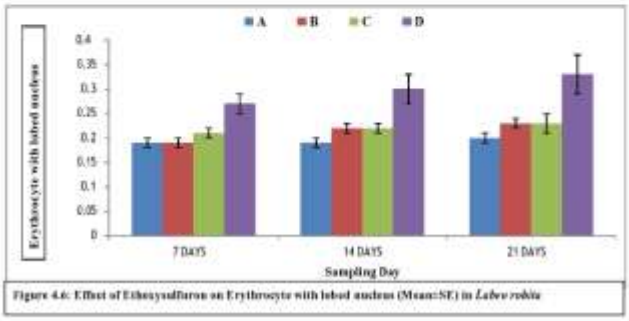


Figure 4.6: Effect of Ethoxysulfuron on Erythrocyte with lobed nucleus (Mean±SE) in *Labeo rohita*

Erythrocyte with blabbed nucleus (%): The percentages of fish erythrocytes with blabbed nuclei that were present in the treatment groups shown in **figure (4.8).** The findings showed that after 7, 14 and 21 days of Ethoxysulfuron poisoning, the percentages of erythrocytes with blabbed nuclei in fish from Group C and D considerably (P≤0.05) increased erythrocytes with a blabbed nucleus value.

Erythrocyte with vacuolated nucleus (%): The percentages of fish erythrocytes with vacuolated nuclei that were present in the treatment groups show **figure (4.9).** After 7, 14 and 21 days of Ethoxysulfuron poisoning, the percentage of fish in Group C and D with erythrocytes with vacuolated nuclei considerably (P≤0.05) increased.

Erythrocyte with nuclear remnants (%): The percentages of fish-containing erythrocytes with nuclear remains that were present in the treated groups shown in **figure (4.10).** Findings showed after 7, 14 and 21 days of Ethoxysulfuron poisoning, the percentages of erythrocytes with nuclear remains (%) in fish from Group C and D significantly (0.05) increased.

Frequency of DNA damage, isolated hepatocytes, isolated kidney cell, isolated heart cell, isolated Brain cells (%).

In isolated fish gill cells from treated groups, the percentages of DNA damage are shown in **fig.4.11, 4.12, 4.13**. After 7, 14 and 21 days, fish in Groups C and D had significantly ($P \leq 0.05$) higher values of genotoxicity in isolated gills, liver, kidney, heart and brain cells respectively.

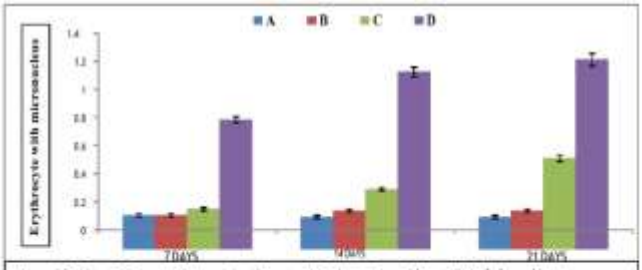


Figure 4.7: Effect of Ethoxysulfuron on Erythrocyte with micronucleus (Mean±SE) in *Labeo rohita*

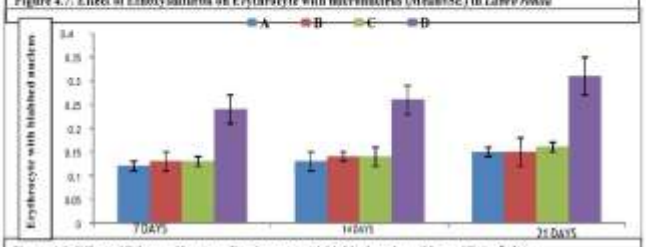


Figure 4.8: Effect of Ethoxysulfuron on Erythrocyte with blabbed nucleus (Mean±SE) in *Labeo*

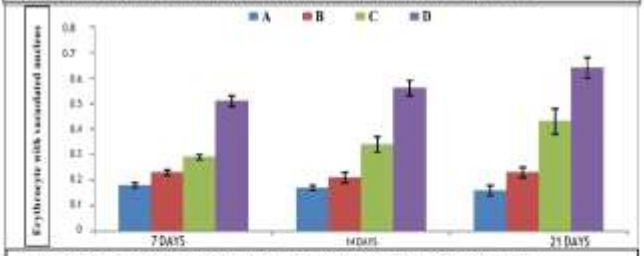


Figure 4.9: Effect of Ethoxysulfuron on Erythrocyte with vacuolated nucleus (Mean±SE) in *Labeo rohita*

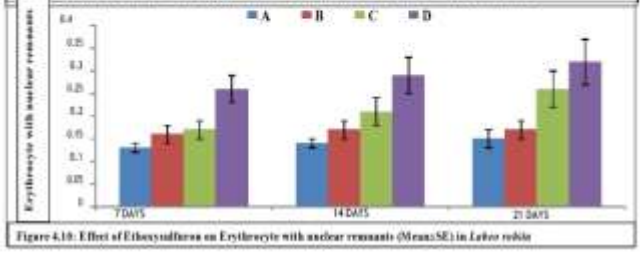
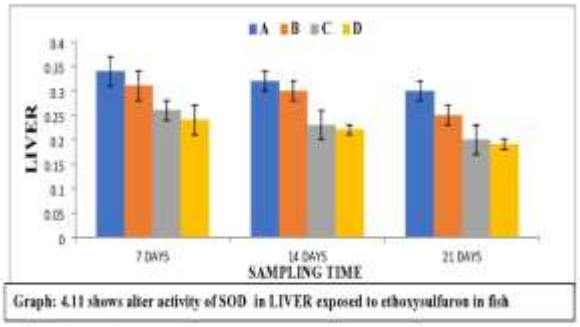


Figure 4.10: Effect of Ethoxysulfuron on Erythrocyte with nuclear remnants (Mean±SE) in *Labeo rohita*



Graph: 4.11 shows alter activity of SOD in LIVER exposed to ethoxysulfuron in fish

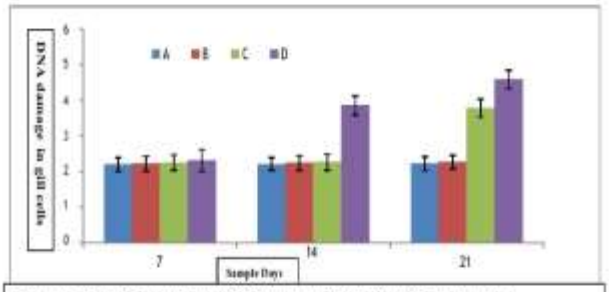


Figure 4.12: Effect of Ethoxysulfuron on DNA damage in gill cells (Mean±SE) in *Labeo rohita*

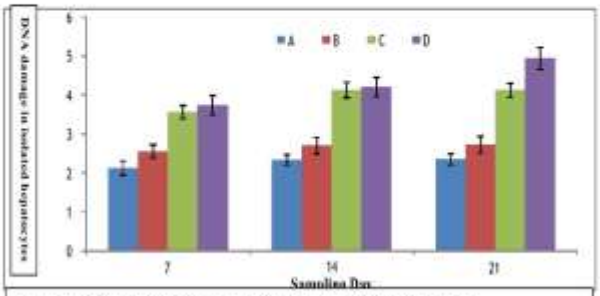


Figure 4.13: Effect of Ethoxysulfuron on DNA damage in isolated hepatocytes (Mean±SE) in *Labeo rohita*

Stress Biomarkers

The results showed that Ethoxysulfuron pesticide exposure caused a significant increase in oxidative stress markers and a decrease in antioxidant enzyme activities in blood and visceral organs**, Figure 4.14 and 4.15.**

Gills oxidative stress parameters

During trial days 7, 14, and 21, it was discovered that fish in groups C treated with 0.375 g/L Ethoxysulfuron and group D treated with 0.50 g/L Ethoxysulfuron both had considerably higher reactive oxygen species levels. After days 7, 14, and 21 of the study, fish in groups C treated with 0.375 g/L Ethoxysulfuron and group D treated with 0.50 g/L Ethoxysulfuron both showed significantly higher levels of thiobarbituric acid reactive chemicals. After days 7, 14, and 21 of the study, the fish in groups C treated with 0.375g/L Ethoxysulfuron and group D treated with 0.50g/L Ethoxysulfuron both showed substantially reduced glutathione content.

Antioxidant enzymes in gills:

In the fish in groups C treated with 0.375g/L Ethoxysulfuron after days 7, 14, and 21 of the experiment, as well as in group D after days 7, 14, and 21, it was observed that superoxide dismutase levels in the gills had dramatically decreased. After days 7, 14, and 21 of the study, it was shown that catalase levels in the liver were dramatically decreased in both group D and group C fish exposed to 0.375 g/L Ethoxysulfuron. In the fish of the groups after days 7, 24, and 21, peroxidase was observed to have drastically decreased.

Reactive Oxygen Species and Antioxidant Enzymes in the Liver.

During experimental days 7, 14, and 21, fish from groups C (0.375 g/L) and D (0.50 g/L) treated with Ethoxysulfuron had substantially higher concentrations of ROS and TBARS in their livers ($P \leq 0:05$). After days 7, 14, and 21 of the experiment, the GSH, SOD, CAT, and POD concentrations in the liver of fish in groups C and D treated with 0.375 g/L and 0.50 g/L Ethoxysulfuron, respectively, decreased considerably ($P \leq 0:05$).

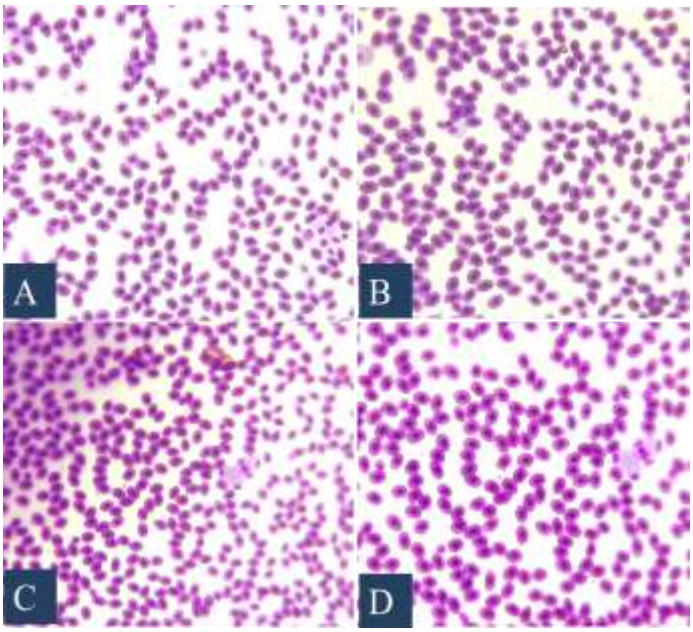


Figure4.14: Showing Erythrocytes Nuclear changes in fish at day 21 of experiment in different groups when Labeo rohita treated with different concentration of Ethoxysulfuron (A) 0.25g/L (B group) irregular shaped erythrocytes. (B) 0.375g/L (C group) blabbed nuclei. (C & D) 0.5g/L (D group) micronuclei, pear shaped erythrocytes, lobed nucleus and vacuolated nucleus.

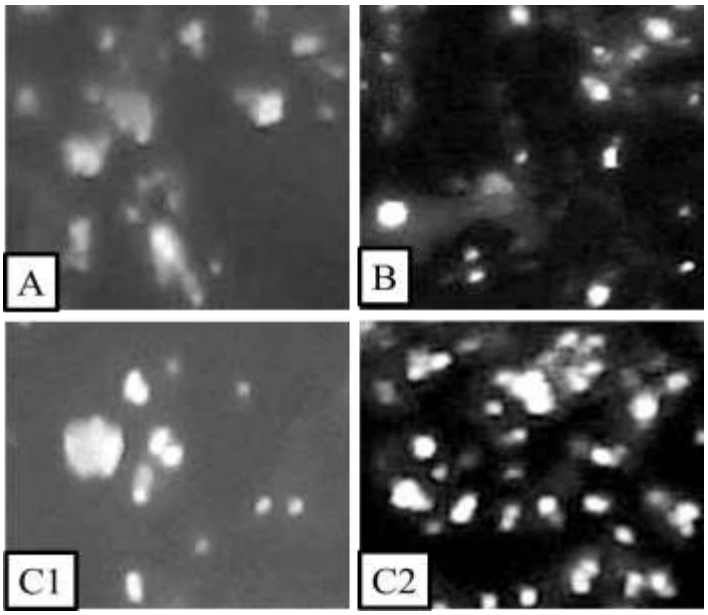


Figure: 4.15: Comet essay showing DNA damage in fish at day 21 of experiment in different groups when Labeo rohita treated with different concentration of Ethoxysulfuron (A) 0.25g/L (B group). (B) 0.375g/L (C group). (C1 & C2) 0.5g/L (D group).

4. Discussion

The severity of the harm caused by pesticides used in the aquatic environment must be evaluated. Herbicides applied to the vegetation surrounding fish ponds significantly alter the morphology, genotoxicity, and nuclear abnormalities in the exposed fish [16]. Herbicides, fungicides, and insecticides from various sources frequently encroach on aquatic environments. Because of their importance to both the economy and the environment, fish are a suitable species for studying the effects of harmful compounds, according to previous studies [17, 18]. Changes in genotoxicity and cellular levels were the most delicate biological reactions associated with fish exposure to aquatic pollutants, according to previous research [19]. It is well known that erythrocyte morphological and genetic variations serve as helpful oxidative stress indicators. Earlier studies have shown that erythrocytes are accurate markers of oxidative stress, displaying a variety of nuclear and morphological amendments in animals visible to numerous hazardous chemicals [20]. These cellular changes might be the result of increased cellular production of reactive superoxide and nitrogenous species [21].

Oxidative damage caused due to various intrinsic as well as extrinsic factors in animals and may result from imbalance between ROS generation and activity of antioxidant defenses. It is possible that toxicants react with water and produce superoxide and it cause greater oxidative damage in freshwater fish [22]. To maintain the balance between oxidative damage and cellular protein to prevent formation of ROS, both enzymatic and non-enzymatic antioxidants are crucial [23]. CAT, SOD, POD and glutathione reductase are all example of cellular protein or antioxidant enzymes that help in inhibiting oxidative stress and their activities, which are usually measured in evaluate the imminent peril of pesticides and herbicides to aquatic organisms [24]. Glutathione reductase has been identified as a valuable indicator for evaluating impact of pesticides and herbicide on aquatic organisms [25]. The value of TBARS and ROS significantly increase in higher days dose in liver and kidney in treated group as compare to control group and the value of GSH, POD, CAT and SOD significantly decrease contrast to control group [26].

Recorded studies have also identified changes in the liver peroxisomal catalase in fish manifest to pesticides and suggesting that peroxisomal catalase may be informative cue for detecting hepatotoxicity caused by toxicant [27].

An abundance of cyclase DNase, which is in charge of the cleaving of extracellular matrix, atomic, and aneuploid polypeptides in addition to oxidative stress - induced to the mitochondrion, could be the cause of the greater proportion of erythrocytes in our study with chromosomal aberrations and lobed nuclei [28]. A number of studies have also discovered morphological and nuclear alterations in the erythrocytes of several fish species, including Cyprinus Carpio and *Labeo rohita* (Ghaffar, 2021), (Ghaffar, 2018), (Hussain, 2014), (Ghaffar, 2015), (Camacho, 2020). According to (Hussain, 2022), increasing lipid peroxidation causes red blood cell walls to become more permeable and less symmetric, which increases osmotic delicacy and causes defects on the surface of red blood cell [29].

In our study, isolated cells from the heart, brain, kidney, gills, and liver of *Labeo rohita* showed a high frequency of DNA damage when tested using a comet assay caused by Ethoxysulfuron. Single-cell gel electrophoresis is responsive, suitable, trustworthy besides widely used for evaluating genotoxicity in aquatic and land-dwelling species [30]. No information about DNA damage

caused by Ethoxysulfuron in the tissues of freshwater fish *Labeo rohita* was previously available in published literature. In our research, we discovered that *Labeo rohita* had significantly more DNA damage. It's possible that a rise in free radical production, which results in oxidative stress, is the cause of genotoxicity in *Labeo rohita.* Additionally, oxidative stress is the main cause of the genotoxic effects [31]. In a number of animal tissues, recent research has shown that the production of oxidative stress and the formation of free radicals are the primary causes of DNA damage [32].

5. CONCLUSION

According to the findings of this study, Ethoxysulfuron was found to have negative impacts on various organs of *Labeo rohita* fish. The exposure of specimens to various assiduousness of Ethoxysulfuron (0.250g/l, 0.375g/l and 0.5 g/l) resulted in harmful effects on cells of the blood, brain, gills, liver, and kidneys. Erythrocyte abnormalities in freshwater fish *(Labeo rohita*) treated with Ethoxysulfuron showed considerably ($p<0.05$) greater morphological level and nuclear abnormalities. DNA damage was considerably elevated in isolated fish cells from the treated fish's gills, liver, brain, kidney, and heart. Comet assays are accurate methods for assessing genotoxicity biomarkers. The findings of the present study will highlight a significant problem with regard to human health that may be brought on by aquatic animals, particularly *Labeo rohita*, which are extremely valuable commercially and essential to human nutrition, being exposed to Ethoxysulfuron poisons. longer time exposure to Ethoxysulfuron decrease the activity of antioxidant enzyme defensive system and caused oxidative stress of fish badly.

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